



Effect of *Lactobacillus* and *Bifidobacterium* on *Cryptosporidium parvum* oocyst viability

James C. Foster^a, Matthew D. Glass^a, Polly D. Courtney^b, Lucy A. Ward^{a,*}

^aDepartment of Veterinary Preventive Medicine, Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Veterinary Medicine, The Ohio State University, Wooster, OH 44691, USA

^bDepartment of Food Science and Technology, College of Food, Agriculture and Environmental Sciences, The Ohio State University, Columbus, OH 43210, USA

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Abstract

Cryptosporidium parvum, an opportunistic protozoan parasite of humans and animals worldwide, causes diarrheal disease that is typically self-limiting in immunocompetent hosts but often life-threatening to immunocompromised individuals. Clinical management of cryptosporidiosis has been hampered by the lack of effective anti-cryptosporidial drugs. In vivo studies in mice have demonstrated that administering live bacterial cell supplements (probiotics) prior to challenge with *C. parvum* reduces the severity and duration of symptoms associated with cryptosporidiosis. It has been suggested that probiotics may inhibit infection through excretion of substances harmful to one of the parasite's developmental stages and possibly offer new therapeutic agents for the treatment of cryptosporidiosis. We evaluated the in vitro effect of four common food fermenting bacteria derived from human intestines (*Lactobacillus acidophilus*, *L. reuteri*, *Bifidobacterium breve* and *B. longum*) on the oocyst (infective) stage of *C. parvum* using a novel flow cytometric viability assay. Compared to broth controls, *Lactobacillus* supernatants significantly reduced oocyst viability up to 81% whereas *Bifidobacterium* supernatants reduced viability only 10–37%. These results suggest the presence of anti-microbial substance(s) against the oocyst stage of *C. parvum* in the supernatants of *L. reuteri* and *L. acidophilus* broth cultures. Further study is needed to delineate the nature and identity of the factor(s) involved.

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1. Introduction

Cryptosporidium parvum is a water-borne protozoan parasite that causes acute diarrhea in humans and domestic animals (Heine et al., 1984). The *C. parvum* oocyst is long-lived and resistant to standard water disinfection treatments. Infection occurs when oocysts in food, water, or the environment are ingested. Exposure to reducing conditions or pancreatic enzymes and bile salts cause oocysts to excyst and release four sporozoites that invade the host's intestinal epithelium and undergo both asexual and sexual multiplication cycles. The life cycle produces new oocysts that are shed in large quantities in the feces. In experimental studies in gnotobiotic pigs, it was shown that ingestion of a single

viable oocyst can cause moderate to severe diarrhea, dehydration, weight loss, and even death (Pereira et al., 2002). Management of cryptosporidiosis, the disease caused by *C. parvum* infection, has been hampered by the lack of effective drugs.

Probiotics are viable non-pathogenic micro-organisms that, when ingested, have beneficial effects in the prevention and treatment of pathological conditions (Duggan et al., 2002; Rolfe, 2000). General mechanisms of action that have been prescribed to probiotics include competition for receptor sites on the intestinal surface, immune system stimulation, excretion of anti-microbial substances, and competition with pathogens for intraluminal nutrients (Duggan et al., 2002; Rolfe, 2000). *Lactobacilli* and *Bifidobacteria* are lactic acid bacteria (LAB) commonly found in fermented dairy products such as yogurt which exhibit probiotic properties (Canganella et al., 1998; Duggan et al., 2002; Tejada-Simon et al., 1999). Antimicrobial compounds produced

*Corresponding author. Tel.: +1-330-263-3744; fax: +1-330-263-3677.

E-mail address: ward.195@osu.edu (L.A. Ward).

by LAB can inhibit growth of strains of *E. coli*, *Pseudomonas* spp. and *Listeria monocytogenes* in vitro (Kanatani et al., 1995; O'Riordan and Fitzgerald, 1998; Zamfir et al., 1999). Furthermore, numerous in vivo human and animal studies have clearly illustrated the preventative or therapeutic potential that LAB have for a variety of gastrointestinal diseases (Duggan et al., 2002; Isolauri, 2001). In fact, *Lactobacillus acidophilus* and *L. reuteri* have been shown to reduce the duration and number of *C. parvum* oocysts shed in feces of experimentally infected mice (Alak et al., 1997; Alak et al., 1999; Waters et al., 1999) which suggests that certain LAB may possess potential therapeutic properties against *C. parvum*.

Although animal studies probably remain the best definitive test system by which *C. parvum* viability and infectivity should be evaluated (Bukhari et al., 2000), delineating the specific mechanisms by which LAB confer their effects against *C. parvum* in vivo is quite difficult. Animal studies are also problematic in that the number of oocysts needed for infection varies between animal species (i.e. hundreds to thousands of oocysts for mice vs one oocyst for germ-free pigs) and different animal species are differentially susceptible to infection by the human and bovine *C. parvum* genotypes (i.e. germ-free pigs can be infected with both genotypes whereas mice are resistant to infection by human genotype 1) (Pereira et al., 2002). Finally, the time, cost and responsibilities associated with maintaining animals, especially large animals, are impractical for most laboratories. In contrast, in vitro systems can be manipulated with relative ease to determine the presence or absence of selected LAB effector mechanisms. One of the more recent and efficient in vitro methods for assessing oocyst viability has been achieved through the use of fluorogenic vital dyes, the most popular being 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Campbell et al., 1992; Schupp and Erlandsen, 1987). In this procedure, viable oocysts absorb DAPI and exclude PI while non-viable oocysts stain with PI. Schupp and Erlandsen (1987) clearly showed *Giardia muris* oocyst viability as determined by PI staining or exclusion strongly correlated with infectivity in a mouse model for giardiasis, and Campbell et al. (1992) demonstrated that the DAPI-PI method correlated well with *C. parvum* oocyst excystation. Dowd and Pillai (1997) incorporated PI staining with an indirect fluorescent antibody detection method thereby providing the advantage of both oocyst detection and viability determination at the same time in the same sample. Unfortunately, these fluorogenic vital dye assays require considerable microscopic evaluation time, which limits analysis to a few hundred or fewer oocysts per sample. Thus, we adapted the vital dye assay and fluorescent antibody detection method to the flow cytometer, thereby permitting fast, consistent

analysis of the viability of large quantities of *C. parvum* oocysts. We report here the effect of bacterial cell-free supernatants from *Lactobacillus acidophilus*, *L. reuteri*, *Bifidobacterium breve* and *B. longum* broth cultures on *C. parvum* oocyst viability using this assay.

2. Materials and methods

2.1. *Cryptosporidium parvum*

Freshly propagated bovine (genotype 2) *C. parvum* oocysts from experimentally infected neonatal calves were used for this study. *C. parvum* strain OH was originally obtained from an infected adult laboratory worker by Dr. Lucy Ward, OSU/OARDC, Food Animal Health Research Program, Wooster, OH, and has been maintained by passage in neonatal calves. The OH strain was confirmed as bovine genotype 2 by nested PCR-RFLP of the small subunit ribosomal RNA gene (Xiao et al., 1999) before and after calf passages. Oocysts were purified from the feces of OH-infected calves within 2 weeks of infection by sodium chloride (NaCl) and cesium chloride (CsCl) gradient density centrifugation. Briefly, day-old male calves were placed in metabolic cages, orally infected with 10^6 – 10^8 OH oocysts, and monitored daily for oocyst shedding by ultraviolet/acid-fast staining (UV/AF) of prepared fecal swabs (Nielsen and Ward, 1999). All UV/AF positive feces were collected daily and stored at 4°C. Approximately 5 l of feces collected during the first week post-shedding were subsequently pooled and concentrated by centrifugation at 3000g for 15 min at 4°C to remove urine and excess fluid. The remaining fecal material was diluted 1:2 in saturated NaCl and centrifuged at 1000g for 10 min at 4°C. The resulting supernatant was saved, diluted 1:3 with 0.2 micron filtered water and centrifuged at 3000g for 15 min at 4°C. The pellet containing oocysts was then washed sequentially in 1.0% sodium hypochlorite solution, 0.1% sodium thiosulfate solution, and Alsever's solution at 2000g for 10 min at 4°C. After the final wash, the pellet was suspended in 2 ml of cold Tris buffer (50 mM Trizma Base and 10 mM EDTA, Sigma, St. Louis, Missouri, USA), layered over a preestablished CsCl gradient [3 ml of 1.4 g ml⁻¹ CsCl (bottom), 3 ml of 1.15 g ml⁻¹ CsCl (middle), and 3 ml of 1.05 g ml⁻¹ CsCl (top)] and centrifuged (16 000g, 1 h, 4°C). A visible band containing oocysts in the uppermost layer was removed and washed 3 × with cold water (2000g, 10 min, 4°C). After the final wash, oocysts were suspended in 5 ml of 0.2 micron filtered water. The oocyst concentration in the test pool was determined using a hemocytometer, adjusted to 5×10^6 oocysts/ml in water, and stored at 4°C.

2.2. Bacterial strains and supernatants

Lactobacillus reuteri American Type Culture Collection (ATCC) 23272 and *L. acidophilus* NCFM™ or ATCC 700396 (Sanders and Klaenhammer, 2001) strains were used for this study. Both strains were originally isolated from human feces. *Bifidobacterium breve* ATCC 15698 strain, which was originally isolated from an infant, and *B. longum* ATCC 15707, originally isolated from an adult human, were also used in the study. Lactobacilli (MRS) broth and reinforced clostridial medium (RCM) (Difco, Sparks, Maryland, USA) were used for inoculation of frozen cultures and propagation of *Lactobacillus* and *Bifidobacterium*, respectively. Cells were grown overnight at 37°C anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in anaerobic chambers. Cultures were subcultured once, and then inoculated into 10 ml of their respective medium. After reaching stationary phase (24 h), cells were removed from broth cultures by centrifugation at 6500g at 4°C for 10 min. Supernatants were filtered with a 0.45 µm membrane. One-milliliter aliquots were placed in microcentrifuge tubes and stored at –70°C until assayed.

2.3. Viability assay

Aliquots of oocysts (5×10^5) were suspended in test bacterial cell supernatants (undiluted or diluted 1:2, 1:5, 1:10, 1:100 in water) and placed in wells of a V-bottom 96-well plate (Fisher Scientific, Hanover Park, Illinois, USA). Positive control wells contained 5×10^5 oocysts in either MRS or RCM broth diluted in water as above. Negative controls consisted of MRS or RCM broth diluted in water as above. Wells containing the oocysts and test supernatants or broth controls were prepared in triplicate in 200 µl volumes and incubated at 37°C for 24 h. Oocysts in each well were then pelleted (2000g, room temperature, 10 min), washed in phosphate buffered saline, pelleted, and subsequently labeled with FITC-conjugated monoclonal antibody as suggested by the manufacturer (A400FL Crypt-a-Glo™ Waterborne Inc, New Orleans, Louisiana, USA). In addition, 5×10^5 oocysts from the original 4°C test pool were prepared in triplicate wells and labeled with the FITC-monoclonal antibody to serve as the oocyst viability control. All FITC-labeled oocysts (5×10^5) were then transferred to 12 × 75 mm Falcon polystyrene test tubes (Becton Dickinson, Mansfield, Massachusetts, USA), brought to a volume of 1 ml with deionized water, and 5 µl of PI (1 mg/ml in PBS, pH 7.2, Sigma) was added to each tube and incubated for 5 min at 37°C (Campbell, 1992). Immediately following PI-staining, oocyst preparations were subjected to flow cytometric analysis using a Becton Dickinson FACS Caliber with a 488 nm air-cooled argon laser (BD Biosciences, San Jose,

California, USA). Logarithmic signals were used for all parameters. The forward angle light scatter (FSC) was set at E00, the green fluorescence detector (FL1) was set at 682 V, and the red fluorescence detector (FL2) was set at 606 V. The FSC detector was used as the threshold for all samples and set to a value of 50. Oocysts were gated on the FSC by FL1 dot plot and the debris was removed using the Paint-A-Gate Pro Power software (Version 3.0, 1996, Becton Dickinson). Using an FSC by FL2 dot plot the gated oocysts population from the FSC by FL1 dot plot was divided into two separate populations. The population recorded to have red fluorescence was non-viable, and the other population was considered viable.

2.4. Statistical analysis

The percent viable oocysts in treatment and controls were compared using One-way ANOVA (Software Analysis Systems Institute, North Carolina, USA). When ANOVA identified significant differences, a Tukey test for multiple comparisons was used to identify samples that were significantly different. Significance was assessed at $P \leq 0.05$. Data are reported as percent of non-viable cells + SEM for each dilution of supernatant and broth. The percent reduction in viability by the bacterial supernatants compared to their respective broth controls were calculated as: $X = 100 - [((\% \text{ viable in bacterial supernatant wells}) \times (100)) / (\% \text{ viable in broth control wells})]$.

3. Results

Oocysts labeled with the FITC conjugated monoclonal antibody were successfully separated from the sample debris on FSC × FL1 dot plot allowing for easy gating of the parasite populations for subsequent determination of viability (Fig. 1A and C). Staining of oocysts by PI reached maximum by 5 min and easily allowed for determination of the percent non-viable oocysts in the FITC-labeled population. Oocysts that did not exclude PI fluoresced red on the FSC × FL2 dot plot were considered non-viable (Fig. 1B and D). Oocysts that excluded PI (and hence did not fluoresce red) were considered viable (Fig. 1B and D).

Viability of the oocyst test pool was >95% (Fig. 1A and B). Preliminary studies indicated that oocyst viability of >90% was needed to ensure consistency and reproducibility of the assay. Both the bacterial cell-free supernatants and their broth controls were effective in reducing oocyst viability (19–86% for *Lactobacillus* supernatants vs 21–56% for MRS broth controls, Fig. 2; and 25–71% for *Bifidobacterium* supernatants vs 37–53% for RCM broth controls, Fig. 3). In fact, the mean number of non-viable oocysts for each dilution of

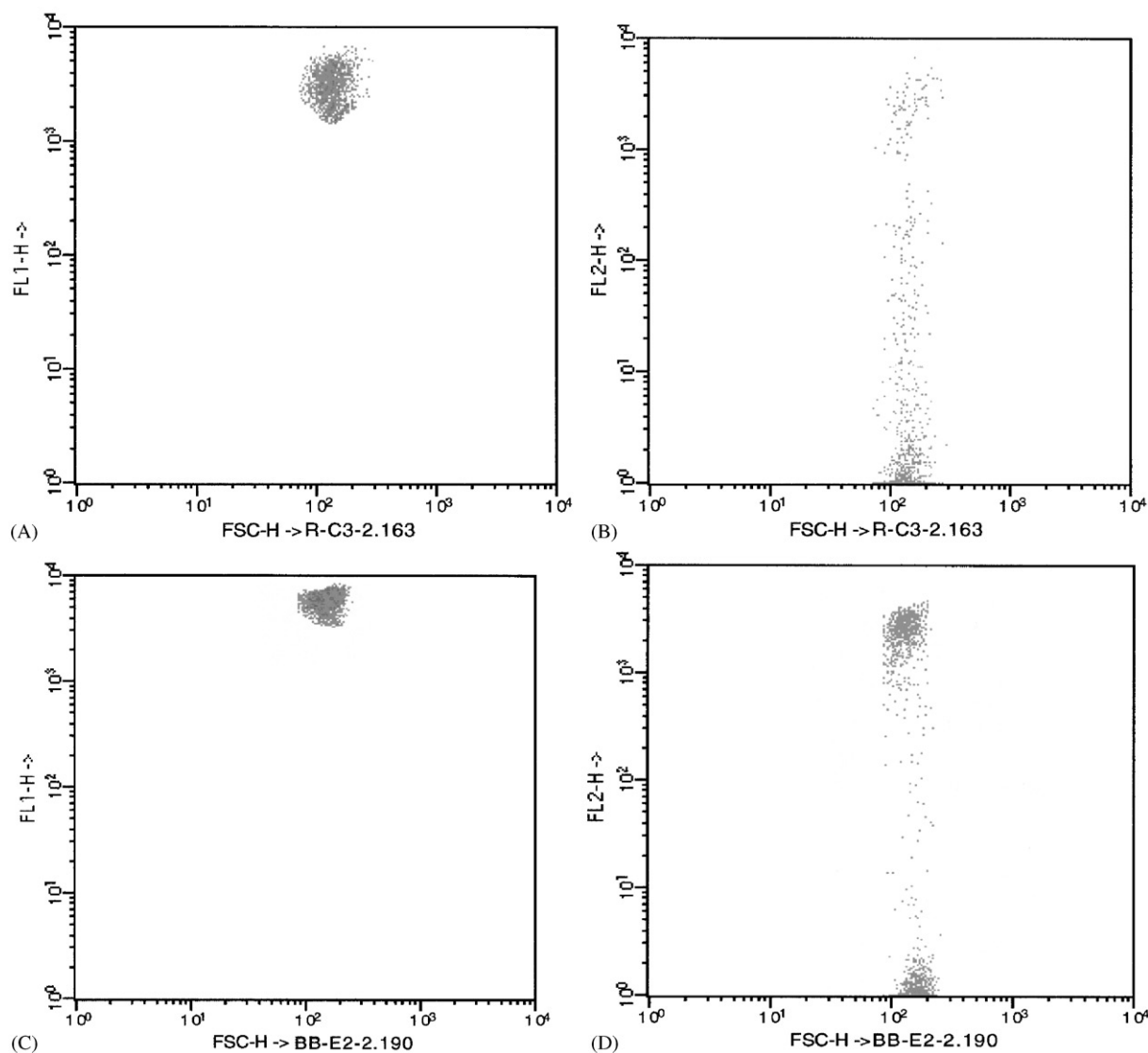


Fig. 1. (A) FSC \times FL1 dot plot of *C. parvum* oocysts from the 4°C oocyst test pool (*C. parvum* viability control). Oocysts identified by FITC-labeled monoclonal antibody (green fluorescence). (B) FSC \times FL2 dot plot of the gated oocyst population in (A). Non-viable oocysts are unable to exclude PI stain (red fluorescence). Note that only a few non-viable oocysts are present in the test pool. (C) FSC \times FL1 dot plot of oocysts after 24-h incubation in bacterial cell-free supernatant of *B. breve*. Oocysts identified by FITC-labeled monoclonal antibody (green fluorescence). (D) FSC \times FL2 dot plot of the gated population in (C). The non-viable population separates from the viable population due to staining by PI (red fluorescence).

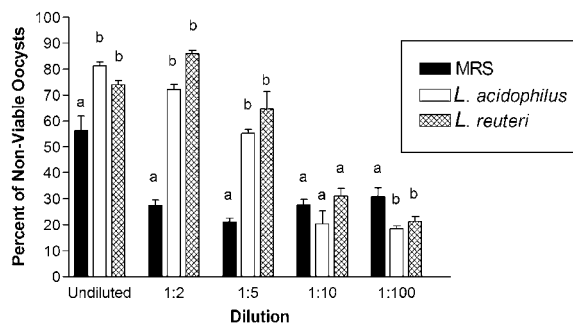


Fig. 2. Percent of non-viable oocysts as determined by FACS analysis after incubation with *Lactobacillus* spp. cell supernatants or MRS broth. Bars with differing superscripts at a given dilution are significantly different (Tukey test, $P < 0.05$).

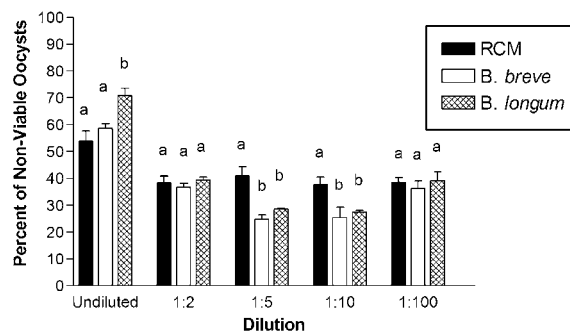


Fig. 3. Percent of non-viable oocysts as determined by FACS analysis after incubation with *Bifidobacterium* spp. cell supernatants or RCM broth. Bars with differing superscripts at a given dilution are significantly different (Tukey test, $P < 0.05$).

Table 1
Percent reduction in *C. parvum* oocyst viability by LAB cell-free supernatants

Dilution	<i>L. acidophilus</i>	<i>L. reuteri</i>	<i>B. breve</i>	<i>B. longum</i>
Undiluted	56 ± 7 ^a	39 ± 8 ^a	10 ± 17	37 ± 4 ^a
1:2	61 ± 6 ^a	81 ± 1 ^a	0.2 ± 0.3	4 ± 5 ^b
1:5	43 ± 2 ^a	55 ± 16 ^a	0 ± 0 ^b	0 ± 0 ^b
1:10	0 ± 0 ^b	5 ± 9 ^b	0 ± 0 ^b	0 ± 0 ^b
1:100	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	2 ± 4 ^b

Values with different superscripts are significantly different ($P < 0.05$).

bacterial supernatants and its broth control were significantly different than the mean percent of non-viable oocyst in the original 4°C test pool; hence, comparisons for statistical analysis were only made between the bacterial cell supernatant and its respective broth control.

The undiluted, 1:2, and 1:5 dilutions of both *Lactobacillus* supernatants significantly reduced the percentage of viable *C. parvum* oocysts compared to the MRS broth controls ($P \leq 0.05$; Table 1, Fig. 2). There were no significant differences in the percent reduction in oocyst viability between the *L. acidophilus* and *L. reuteri* supernatants ($P \geq 0.05$; Table 1, Fig. 2). Incubation with supernatants from the *Bifidobacterium* species was less successful at reducing oocyst viability with only the undiluted supernatants of *B. longum* resulting in a significant reduction in oocyst viability compared to the RCM broth controls ($P < 0.05$; Table 1; Fig. 3). *B. breve* supernatants did not decrease oocyst viability at any concentration compared to the RCM broth controls ($P \geq 0.05$; Table 1; Fig. 3).

4. Discussion

In this study, select species of *Lactobacillus* and *Bifidobacterium* were used to determine their effects on the viability of *C. parvum* oocysts in vitro. Selection of these bacteria was based on their use in a variety of fermented dairy products (Duggan et al., 2002) as well as their presence in the normal microflora of humans (Reuter, 2001) and their ability to resist stimulated small intestinal transit (Charteris et al., 1997). LAB can produce several chemical substances that may work collectively or singly to exhibit antimicrobial properties, or substances from different LAB species may interact with one another to effect anti-microbial properties against a pathogen. Only the antimicrobial properties of individual species of LAB against *C. parvum* oocyst viability were investigated in the present study. Additional studies are underway to define the relationship between LAB effect on oocyst viability and LAB effect on oocyst infectivity in cell culture and germ-free pigs as recent reports suggest fluorogenic vital dye assays overestimate actual oocyst infectivity, particularly for

aged oocysts (Bukhari et al., 2000; Slifko et al., 1997; Slifko et al., 1999).

Reduction of oocyst viability by the broth controls was not unexpected as such growth mediums contain proteolytic digests of proteins as a nitrogen base and residual enzymes could cause excystation of the oocysts. Once excysted, the oocyst will not be able to exclude the PI stain and will hence be considered 'non-viable', and excysted sporozoites are relatively short-lived (<30 min at room temperature in water; Ward, unpublished). However, the finding that the 1:100 MRS broth control and 1:5 and 1:10 RCM broth controls reduced viability over that of their respective bacterial cell supernatants was unexpected and its cause is unknown. It is plausible that certain compounds that are toxic to oocysts in the broth mediums are also utilized by the bacteria. Thus, such compounds may be present in concentrations great enough to exert oocyst toxicity at the tested dilutions of the broths but are absent from the tested dilutions of the bacterial cell supernatants.

The finding that supernatants from both *L. acidophilus* and *L. reuteri* significantly increased the percent of non-viable oocysts over that of their respective MRS broth controls, however, suggests the presence of additional factors from these *Lactobacillus* spp. that are capable of inactivating *C. parvum* oocysts. Lactic acid, acetic acid, ethanol and hydrogen peroxide are the most widely produced antimicrobial substances but are not specific for the organisms they affect (Zamfir et al., 1999). *Lactobacillus reuteri* also produces a broad-spectrum antimicrobial substance during fermentation called reuterin (Talarico et al., 1988). Specific antimicrobial compounds have also been identified with LAB; *L. casei* produces peptides selectively active against *Salmonella typhimurium* both in vitro and in vivo (Coconnier et al., 1998), and *L. acidophilus* IBB801 produces acidophilin 801 which inhibits *Escherichia coli* and *Salmonella panama* growth in vitro (Zamfir et al., 1999).

Although neither *Bifidobacterium* supernatant was as successful as the *L. acidophilus* and *L. reuteri* supernatants in reducing oocyst viability over that of the broth control, these bacteria may still have potential for therapeutic use against *C. parvum* as suggested by the ability of the undiluted *B. longum* supernatant to significantly reduce oocyst viability compared to the undiluted RCM broth. Thus, *B. longum* may produce the same or similar substance(s) as *L. acidophilus* and *L. reuteri* but in lesser quantities, or produce substances that are simply less harmful to *C. parvum* oocysts. It is also possible that substances produced by a *Bifidobacterium* species require the presence of other LAB products to exert their anti-microbial properties as evidenced by findings from a recent in vivo study evaluating the effect of feeding bacteria derived from porcine intestinal contents to neonatal swine naturally

infected with *Cryptosporidium* (Rotkiewicz et al., 2001). Administration of *L. acidophilus* or a mixture of *Bifidobacterium* spp. to piglets positively affected the elimination of *Cryptosporidium* oocysts from feces and improved systemic metabolism and composition of the intestinal microflora (Rotkiewicz et al., 2001).

The available literature on the survival of oocysts in association with micro-organisms and the role of biological antagonism is sparse and provides no insight into potential mechanisms by which probiotic bacteria interact with *C. parvum* (Chauret et al., 1998; Heisz et al., 1997; Zuckerman et al., 1997). This study provided evidence to suggest that *L. acidophilus*, *L. reuteri* and *B. longum* produced factors that are detrimental to *Cryptosporidium* oocysts, and lend support to the hypothesis that anti-microbial products may be a mechanism by which probiotics affect *C. parvum*. In addition, the flow cytometric viability assay described herein provides a reliable and relatively rapid assay for screening compounds such as bacterial cell-free supernatants or other chemotherapeutics for anti-cryptosporidial activity. The protective mechanism(s) that these *Lactobacilli* and *Bifidobacterium* confer against *Cryptosporidium* and the nature and identity of the factor(s) involved requires further study both in vitro and in vivo.

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